Molecular Mechanisms Related to Colistin Resistance against Gram Negative Isolates

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Abstract

Polymyxins, a structurally distinct class of non-ribosomal, cyclic oligopeptides antimicrobials, include five chemically distinguished compounds (polymyxins A, B, C, D, and E) of which polymyxin B and colistin (polymyxin E) are the only two polymyxins currently available on the market. In 1947 in Japan, Koyama discovered polymyxins, initially, he had reported the colistin as a secondary metabolite of the Gram-positive soil bacterium Paenibacillus polymyxa subsp. Colistinus. Colistin is an active agent against aerobic Gram-negative pathogens that frequently represent the mainspring of life-threatening infections, such as carbapenem-resistant P. aeruginosa, A. baumannii, K. pneumoniae, E. coli, and other Enterobacterales. Noteworthy, some bacterial species, such as; Serratia marcescens, Proteus spp., Providencia spp., Morganella morganii, Vibrio cholera, Brucella, Campylobacter, Legionella, Chromobacterium, Neisseria spp., Edwardsiella , some Aeromonas species, Burkholderia cepacia, anaerobic Gram-negative cocci, eukaryotic microbes, and mammalian cells, are possessing intrinsic colistin resistance. Antibacterial activity of colistin occurs on the outer membrane (OM) of Gram-negative bacteria. The antibacterial activity of colistin occurs through two-step mechanisms that are initial binding and employed permeabilization of the outer LPS membrane induces the displacement of Ca^{2+} and Mg^{2+} ions from the phosphate groups of LPS in a competitive way resulting in destabilizing cytoplasmic membrane, leading to disruption of the outer LPS and the loss of inner cellular contents, hence bacterial killing. The critical step of colistin action is based on the electrostatic interaction of cationic colistin peptide and anionic lipid A membrane also known as endotoxin component of LPS layer.

Keywords: Colistin Resistance

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Introduction:

Polymyxins, a structurally distinct class of non-ribosomal, cyclic oligopeptides antimicrobials, include five chemically distinguished compounds (polymyxins A, B, C, D, and E) of which polymyxin B and colistin (polymyxin E) are the only two polymyxins currently available on the market. In 1947 in Japan, Koyama discovered polymyxins, initially, he had reported the colistin as a secondary metabolite of the Gram-positive soil bacterium Paenibacillus polymyxa subsp. Colistinus **(1).**

Historically, colistin was first used in the 1950s as an intravenous formulation. In 1959, the US FDA approved colistin as an antimicrobial agent against GNB due to its bactericidal activity for the treatment of various types of infections, including infectious diarrhea and urinary tract infections. Moreover, polymyxins have been administered for several decades in topical formulations for eye and ear infections as well as for selective bowel decontamination. Additionally, polymyxins were used to fight infections caused by intractable GNB. Colistin and polymyxin B have already been used for decades in veterinary medicine for prophylactic and therapeutic purposes **(2).**

Colistin is an active agent against aerobic Gram-negative pathogens that frequently represent the mainspring of life-threatening infections, such as carbapenem-resistant P. aeruginosa, A. baumannii, K. pneumoniae, E. coli, and other Enterobacterales. Noteworthy, some bacterial species, such as; Serratia marcescens, Proteus spp., Providencia spp., Morganella morganii, Vibrio cholera, Brucella, Campylobacter, Legionella, Chromobacterium, Neisseria spp., Edwardsiella , some Aeromonas species, Burkholderia cepacia, anaerobic Gram-negative cocci, eukaryotic microbes, and mammalian cells, are possessing intrinsic colistin resistance **(2).**

On account of the reported adverse events of polymyxins mainly nephrotoxicity and neurotoxicity, alongside to the discovery and approval of new and effective antibiotics, the clinical use of polymyxins was largely abandoned by the mid-1970s. However, they remained in clinical practice for patients suffering from cystic fibrosis (CF) due to pseudomonal lung infections and in topical solutions with other antimicrobial agents for the treatment of ear or eye infections **(3).**

By the mid-1990s the polymyxins had re-emerged as a last-resort treatment against MDR and XDR Gram negatives, not because of an improved safety profile, but rather due to the emergence of XDR Gram-negative superbugs, particularly P. aeruginosa, A. baumannii, and K. pneumonia, which are resistant against all other available antibiotics, besides the lack of novel antimicrobials available to treat MDR bacterial infections **(2).**

Unfortunately, the overuse and misuse of colistin among humans and animals medicine have led to the global emergence of colistin-resistant pathogens. However, the development of bacteria resistant against colistin may also occur unaccompanied by any prior exposure to colistin, leaving clinicians barehanded to treat patients **(4).**

Mechanism of Colistin Activity

Antibacterial activity of colistin occurs on the outer membrane (OM) of Gram-negative bacteria. The antibacterial activity of colistin occurs through two-step mechanisms that are initial binding and employed permeabilization of the outer LPS membrane induces the displacement of Ca^{2+} and Mg^{2+} ions from the phosphate groups of LPS in a competitive way resulting in destabilizing cytoplasmic membrane, leading to disruption of the outer LPS and the loss of inner cellular contents, hence bacterial killing. The critical step of colistin action is based on the electrostatic interaction of cationic colistin peptide and anionic lipid A membrane also known as endotoxin component of LPS layer. Furthermore, it has been reported that bactericidal activity is independent of the passage of colistin into a bacterial cell but inhibited in the presence of these divalent cations. However, LPS is the initial target for bacterial killing, but still, the exact mode of colistin action remains uncertain. Another antibacterial mechanism of colistin occurs by a potent antiendotoxin activity where the lipid A portion of LPS represents an endotoxin in Gram-negative bacteria. Therefore, colistin inhibits the endotoxin activity of lipid A by binding to and neutralizing the LPS molecules. This antibacterial activity mechanism occurs in vivo only. Moreover, another mechanism of action occurs by vital respiratory enzymes (type II NADH-quinone oxidoreductases NADH-2) inhibition by colistin drug in Gram-negative bacteria **(5).**

The alternative strategy of colistin action occurs by induction of rapid cell death via hydroxyl radical production through colistin binding to the lipid membrane. The free radicals are generated when colistin crosses the OM and IM of LPS. The hydroxyl radical generation occurs via the production of the reactive oxygen species; hydroxyl radicals. (•OH), superoxide (O–2), and hydrogen peroxide (H_2O_2), which cause oxidative stress. O–2 is generated when colistin enters into and crosses the OM and IM, followed by the conversion of O–2 into H_2O_2 by superoxide dismutase. After that, H_2O_2 oxidizes ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}), besides the formation of •OH; this process is known as Fenton reaction. This reaction can induce oxidative damage in bacterial DNA, proteins, and lipids, leading to cell death. This mechanism of killing has been shown to occur in the colistin-sensitive and MDR isolates of Acinetobacter baumannii and Escherichia coli but does not take place in polymyxin-resistant strains **(6).**

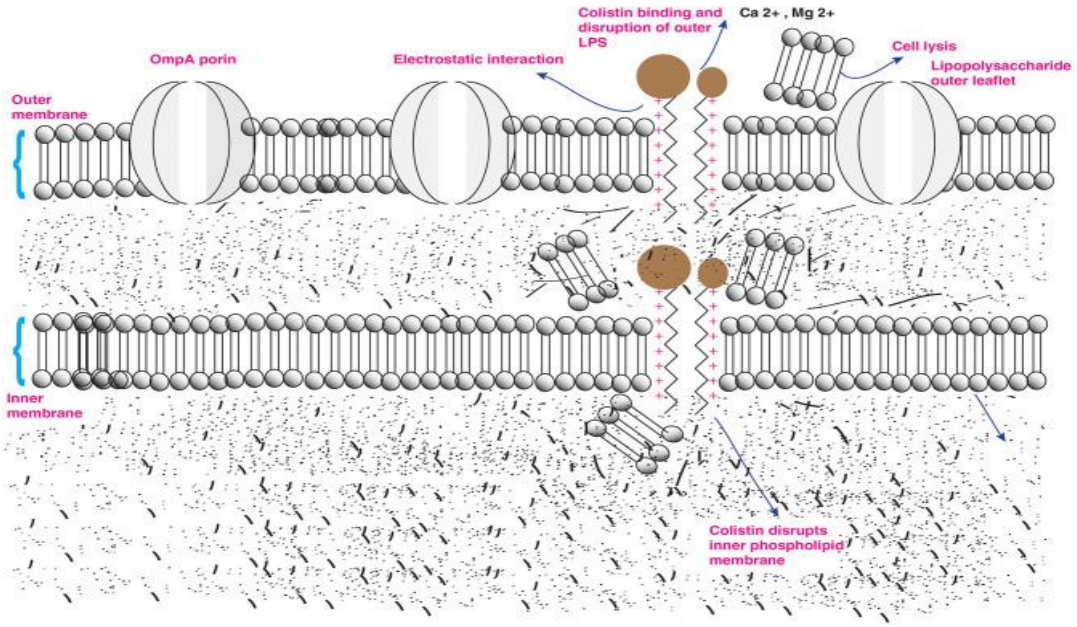

Figure 1. Action of colistin on bacterial membrane. The cationic cyclic decapeptide structure of colistin binds with the anionic LPS molecules by displacing calcium and magnesium from the outer cell membrane of Gram-negative bacteria, leading to permeability changes in the cell envelope and leakage of cell contents **(7)**.

Mechanism of Colistin Resistance

Although the main mechanism of resistance to colistin is unclear, Gram-negative bacteria employ several mechanisms to protect themselves against colistin toward other polymyxins. Most colistin-resistance mechanisms are adaptive mechanisms which occur after in vitro exposure. Resistance to colistin occur with LPS modification via different routes. The most common strategies for resistance to colistin are modifications of the bacterial outer membrane through alteration of the LPS and reduction in its negative charge. The other strategy is the overexpression of efflux-pump systems. Another mechanism is overproduction of capsule polysaccharide. No enzymatic mechanisms of resistance have been reported, but strains of P. polymyxa produce colistinase **(8)**.

Intrinsic resistance mechanisms

Resistance to polymyxins occurs naturally in P. mirabilis and S. marcesens by modification of the LPS via cationic substitution. The mechanism of resistance in these species is linked to expression of the arnBCADTEF operon and the eptB gene. In this way, the 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) cationic groups are added to the LPS by this operon and gene, respectively. It has been shown that the LPS of P. mirabilis contains L-Ara4N and the genome of this bacterium contains the eptC gene, which is mediated to the modification of LPS with PETN **(9)**.

Putative loci in P. mirabilis include the sap operon encoding a transport protein, ATPase gene, and O-acetyltransferase gene, which take part in biosynthesis or transfer of amino arabinose. Also,

the existence of rppA/rppB TCS has been discovered to play a role in activation of the arnBCADTEF operon. Similarly, this operon is responsible for intrinsic resistance to colistin in S. marcescens, as it has been shown that arnB and arnC mutants lead to a reduction insusceptibility to colistin (minimum inhibitory concentration [MIC] from 2,048 to 2 μg/mL) compared to the wild type. This modification of LPS and the increase inits charge give rise to the affinity of colistin decrease for binding to LPS. Therefore, intrinsic resistance has occurred in these species **(10).**

Acquired resistance mechanisms in Enterobacterales

Acquired colistin-resistance mechanisms have been recognized in some members of Enterobacterales such as E. coli, Salmonella spp., Klebsiella spp., and Enterobacter spp., and remain unknown for other bacterial species. Resistance mechanisms are presumed to be linked to chromosomal mutation untransferable via horizontal gene transfer. Only one mechanism of resistance has been identified as a transferable mechanism (plasmid-mediated mcr gene) so far. Many genes and operons play a role in modification of LPS, which in turn leads to colistin resistance. These include: genes and operons responsible for encoding enzymes that have a direct role in LPS modification, such as the pmrC and pmrE genes and the pmrHFIJKLM operon; regulatory two-component systems (TCSs), including PmrAB and PhoPQ, as well as crrAB, which regulates the PmrAB system; the mgrB gene, a negative regulator of TCSs, including PmrAB and PhoPQ; plasmid-mediated mcr genes; and Cpx and Rcs as regulator of upregulation of capsule biosynthesis and activator of the efflux pump KpnEF regulating the PhoPQ system, respectively **(8)**.

- **mgrB gene and regulators of PmrAB and PhoPQ two-component systems**

Some operons and regulators have a role in the modification of LPS by PmrAB and PhoPQ TCSs. The pmrABC operon encodes PmrA (BasR) as a regulator protein, PmrB (BasS) as a cytoplasmic membrane-bound sensor kinase, and PmrC as a putative membrane protein. The addition of L-arabinoseamine (L-Ara4N) to the 1-phosphate or 4′-phosphate group leads to colistin resistance. Generally, L-Ara4N is connected to 4′-phosphate and modifies it while PETN is connected to 1-phosphate. The pmrHFIJKLM operon (also named arnBCDADTEF or pbgPE) and PmrE synthesize L-Ara4N from uridine diphosphate glucuronic acid and fix it to lipid A. The biosynthesis of L-Ara4N depends on the pmr (arn) operon **(11).**

Moreover, under environmental stimulants, such as macrophage phagosomes, the high concentration of iron (Fe^{3+}) and exposure to aluminum (Al^{3+}), as well as acidic pH, leads to activation of PmrB. On the other hand, low concentration of Mg^{2+} or Ca^{2+} leads to activation of phoQ. PmrB activates PmrA by phosphorylation, and PmrA in turns activates regulation of the pmrABC and pmrHFIJKLM operons and the pmrE gene. Subsequently, these operons and genes lead to LPS modification by adding PETN and L-Ara4N to lipid A **(8).**

Mutation of pmrA/pmrB results in upregulation of the pmrABC and pmrFHIJKLM operons and pmrE gene. Mutation within the pmrA and pmrB genes leading to colistin resistance has been described in Klebsiella pneumoniae and Salmonella entericca **(12)**.

On the other hand, the phoPQ TCS encodes PhoP as a regulator protein and PhoQ as a sensor kinase. Under conditions of low magnesium or calcium, acidic PH, or cationic antimicrobial peptide, PhoPQ is activated and protects bacteria. Activated PhoPQ leads to modification of lipid A via two routes: PhoQ activates PhoP by its kinase activity via phosphorylation, which activates transcription of the pmrFHIJKLM operon, followed by modification of lipid A; and PhoP indirectly activates pmrA by bypassing the PmrD connector protein, subsequently activates the transcription of the pmrHFIJKLM operon and synthesizes PETN, which transfers it to lipid A. The mgrB gene encodes a small transmembrane protein of 47 amino acids that exerts negative feedback on the PhoPQ TCS. This protein inhibits the kinase activity of PhoQ, which in turn represses expression of the phoQ gene. Nevertheless, mutation/inactivation of the mgrB gene results in upregulation of the phoPQ operon and subsequent activation of the pmrHFIJKLM operon. Finally, production of L-Ara4N leads to modification of lipid A and colistin resistance **(13)**.

- **CrrAB two-component system.**

The crrAB operon encodes two proteins: CrrA as a regulatory protein and CrrB as a sensor kinase protein. Wright et al described that mutation of crrB leads to colistin resistance in K. pneumoniae. The mutated CrrB protein regulates a crrAB-adjacent gene that encodes a glycosyltransferase-like protein, which in turn leads to modification of lipid A **(14)**.

- **Plasmid-mediated resistance to colistin**

Plasmid-mediated colistin is a significant challenge and global concern, because of easy transfer of colistin-resistance genes to susceptible strains. The mcr genes encode MCR, which are cytoplasmic transmembrane proteins found in GNB. These proteins are phosphoethanolamine (pEtN) transferases conferring resistance to COL by attaching a pEtN moiety to the lipid A of lipopolysaccharide in bacterial cell membrane thereby abolishing the negative charges to which cationic colistin/polymyxins have affinity. The mcr genes are responsible for horizontal transfer of colistin resistance. These plasmid-mediated genes were first reported in E. coli isolated from pigs and meat in China, November 2015. MCR is a member of the PETN enzyme family, and its expression leads to addition of PETN to lipid A. Isolates carrying the mcr1 gene display resistance to colistin without other resistance mechanisms. The existence of mcr1 in isolates is enough for colistin resistance, as isolates carrying this gene displayed a four- to eightfold increase in colistin MIC. Following initial findings, mcr1-mediating transferable colistin resistance has been reported in several regions, including Europe, Asia, the Americas, and Africa. There is a hypothesis that mcr1 originated in animals, particularly pigs and cattle, and subsequently spread to humans **(15)**.

This transmissible gene has been reported from diverse genera of Enterobacterales,kincluding E.coli, Klebsiella spp., Entrobacter spp., Salmonella spp., and Shigella spp.,but mostly from E. coli. Some plasmids containing the mcr1 gene carry other genes that are resistant to other antibiotics, such as β-lactams, aminoglycosides, quinolones, sulfonamides, tetracyclines, and fosfomycin. The mcr gene has also been identified in Enterobacterales isolates, which carry such carbapenemase genes. A novel plasmid-mediated colistin resistance gene, known as mcr2, was reported in 2016 in E. coli. Thereafter, mcr3 and mcr4 genes were discovered. Finally three mobile colistin-resistance genes (mcr6, mcr7, and mcr8) were discovered in 2018 **(16).**

- **Role of regulator RamA**

The ramA locus has three genes: ramA, romA, and ramR. The ramR gene plays a role as a repressor of the ramA and romA genes, researchers showed that increased levels of RamA resulted in LPS modification and increased resistance to colistin **(17).**

- **Role of capsule in colistin resistance**

The role of capsular polysaccharide (CPS) has been demonstrated to be protective against cationic antimicrobial peptides, including colistin. K. pneumoniae is able to release CPS from its surface. The number of capsule layers is related to resistance level. It has been observed that K. pneumoniae with several layers was more resistant to colistin than isolates with few layers **(18).**

There are some regulators of capsule formation, such as Cpx (conjugative pilus expression) and Rcs (regulator of capsule synthesis). Cpx and Rcs also appear to contribute to colistin resistance by activating the efflux pump KpnEF and regulating the PhoPQ TCS, respectively. Furthermore, the ugd gene plays a role in CPS and L-Ara4N biosynthesis in that its phosphorylation is related to the synthesis of capsular and colistin resistance. **(18).**

Recently, the morphology and topography of colistin-resistant bacteria have been found to differ from that of colistin-susceptible cells, which could give us further insight into the genetic mechanisms leading to colistin resistance. An atomic force microscopy study was performed of both colistin-resistant and colistin-susceptible strains at different growth phases. Compared with spherically shaped colistin-resistant bacteria at early and mid-logarithmic phases, susceptible cells were found to be rod shaped with pili present at all phases. The number and length of pili for colistin-resistant cells were greatly reduced **(7).**

In addition, colistin-resistant cells had a greater topographic variability and finer surface texture. In the stationary phase, elongated worm-like cells were more prevalent in the susceptible group versus the resistant group, which showed more heterogeneity among the cells in this phase. Of interest, levels of bacterial outer membrane damage after treatment with colistin were similar for both susceptible and resistant cells, showing the ability of colistin-resistant cells to maintain interaction with the outer membrane **(19).**

Based on these findings, it is evident that specific studies examining the genetic mechanisms behind these morphologic and topographic differences need to be performed, so that we may better understand the resistance associated with colistin. **(19).**

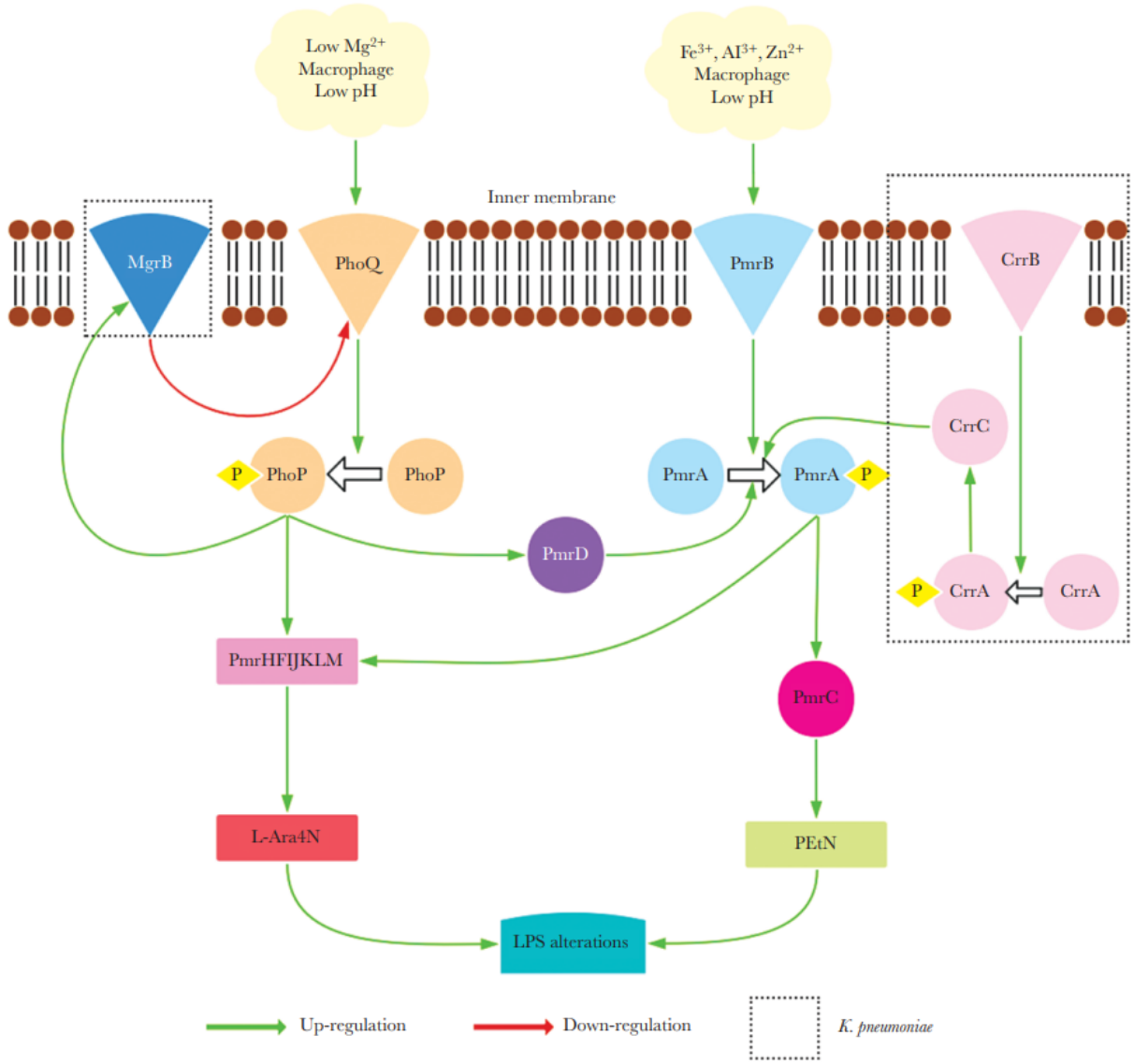

Figure 2. Illustration of Chromosomal Colistin-resistance Mechanisms Multiple mutations contribute to the development of colistin resistance based on subsequent lipopolysaccharide modifications **(20).**

Risk Factors and Prognosis for Colistin resistant bacterial Infection

Previous studies deciphered several factors associated with Colistin resistant bacterial infections and poor treatment outcome. Recently, it has been shown that colistin resistance may arise due to subtherapeutic polymyxin treatment. Except innately Colistin resistant species colistin resistance also was associated with the history of colistin administration. A multicenter study collected colistin-resistant Klebsiella pneumoniae from the bloodstreams of patients and found that previous

treatment with colistin, a preceding colonization of resistant K. pneumoniae, and a Charlson score of ≥3 were correlated with Colistin resistant bacterial infection **(21).**

Colistin-resistant A. baumannii infection or colonization was associated with age and treatment history of carbapenem or colistin. Ventilator support also was determined to be a risk factor of Gram-negative Colistin-resistant microorganism infection. In summary, patients with previous polymyxin exposure as well as those that are critically ill may be at increased risk for colonization or infection with polymyxin-resistant bacteria. Prognosis for patients with infections caused by polymyxin-resistant bacteria also needs to be considered. Colistin resistant bacterial infections in critically-ill patients (including pneumonia, urine tract infection, bacteremia, infection with underlying diseases as transplantation, stroke, chronic obstructive pulmonary disease [COPD], and so on) are associated with high mortality rates (30%–37%). Also, high mortality rates with Colistin resistant bacterial infection were associated with dialysis (63.6%, 7 of 11), septic shock (37.5%–60%, 9 of 24–9 of15), and intraabdominal infection (83.3%, 10 of 12). Patients infected with Colistin resistant bacterial but without previous exposure to colistin would have a significantly better outcome. **(22).**

Mutant Prevention Concentration

Antibiotic resistance is increasingly recognized as a serious global problem. The mutant prevention concentration (MPC) is a novel concept that has been employed in the evaluation of an antibiotic's ability to minimize or limit the development of resistant organisms. The MPC has been defined as the MIC of the least susceptible single-step mutant. By definition, cell growth in the presence of antibiotic concentrations greater than the MPC requires an organism to have developed two or more resistance-causing spontaneous chromosomal point mutations **(23).**

In the 1990s, Baquero suggested that a dangerous concentration range exists in which mutants are selected most frequently. Later the boundaries of the range were defined when mycobacterial mutants recovered from agar plates noticed displaying a characteristic response to fluoroquinolone concentration. Increasing concentration initially causes a sharp drop in colony recovery, as the growth of wild-type cells is inhibited. A distinct plateau is then observed, and finally a second sharp decline in mutant recovery occurs. The plateau arises from the outgrowth of subpopulations of resistant mutants. The second sharp decline takes place when drug concentrations are reached that block the growth of all single-step mutants. Thus, mutants are enriched selectively at fluoroquinolone concentrations between the two sharp drops in colony recovery. This concentration range is termed the mutant selection window **(24)**

The mutant selection window (MSW) is drug concentration between the MIC and MPC drug concentrations. When drug concentrations are below the MIC, neither susceptible nor first step resistant cells are inhibited and as such, there is no selective amplification of resistant subpopulations. For drug concentrations in excess of the MPC, both susceptible and first step resistant cells are inhibited and no selective amplification of resistant subpopulations occur.

Unfortunately, when drug concentrations fall within the MSW, selective amplification of resistant subpopulations occurs as the drug concentration is above the MIC and inhibiting the susceptible cells in the population but not high enough to inhibit resistant subpopulations as the concentration is below the MPC. The lower boundary of the window is the lowest concentration that blocks the growth of the majority of drug-susceptible cells, since below that concentration the mutant cells do not have a growth advantage. The lower boundary can be approximated by the MIC for half the cells in the population ($MIC_{(50)}$); however, inhibition of 99% of the cells ($MIC_{(99)}$) is a more suitable boundary since it is measured more accurately. The standard MIC is less satisfactory in approximating the lower boundary because some selective pressure is exerted when so many cells (10^4 to 10^5) are used in the measurement. Indeed, resistant mutants can be enriched by repeated passage of cells at concentrations just below MIC. Nevertheless, MIC must be near the bottom of the window because treatment of Staphylococcus aureus with moxifloxacin in a dynamic model shows that mutant enrichment occurs above the MIC, not below it (25).

Placing MIC near the lower boundary of the selection window contradicts traditional medical teaching, in which resistant mutants are thought to be enriched selectively at concentrations below MIC. This distinction is important because traditional dosing recommendations to exceed MIC are likely to place drug concentrations inside the selection window where they will enrich resistant mutant subpopulations. Whereas low drug concentrations do not enrich resistant mutants, they do allow pathogen population expansion; consequently, low drug doses indirectly foster the generation of new mutants that will be enriched by subsequent antimicrobial challenge (26).

The upper limit of the window is the drug concentration that blocks the growth of the least susceptible, single-step mutant. Above this concentration, cell growth requires the presence of two or more resistance mutations. Since two concurrent mutations are expected to arise rarely, few mutants will be amplified selectively when a susceptible population is exposed to drug concentrations that exceed the upper boundary. For example, with fluoroquinolones the mutation frequency for resistance due to target (topoisomerase) mutations can be less than 10–7; consequently, more than 10^{14} bacteria would be required to find a cell with two concurrent, independent fluoroquinolone-resistant target mutations. In clinical cases, bacterial populations may reach 10^{10} cells within an infected individual, but 10^{14} is unlikely. Thus, resistance is expected to develop rarely when drug concentrations are kept above the upper boundary of the mutant selection window. This expectation led to the upper boundary being designated the MPC. MPC is approximated experimentally as the lowest concentration that allows no colony growth when more than 10^{10} cells are applied to drug-containing agar plates. The choice of 10^{10} cells is based on several considerations. First, 10^{10} is large enough for mutant subpopulations to be present for testing. Second, infections rarely contain more than 10^{10} organisms. Third, testing more cells is often logistically difficult. In the two cases that have been investigated, a correlation exists between MPC and concentrations that inhibit growth of the least susceptible, first-step mutant (27).

The measurement of MPC is performed in two general ways. In one, cells are applied to multiple agar plates at several antimicrobial concentrations such that the total number of cells tested for a given drug concentration exceeds 10^{10}. When narrow concentration increments are used, isolated colonies can be found and counted to show that their number progressively approaches zero as drug concentration increases (mutant selection curves become steeper as MPC is approached). In a second method, more than 10^{10} cells are placed on single agar plates that differ in drug concentration by two-fold increments. This method, which allows large numbers of isolates to be surveyed, often gives confluent growth or no growth owing to the large concentration increment. With some bacteria, the large inoculum may affect the apparent susceptibility. Correction factors for inoculum effects can be obtained by carrying out the same experiment with smaller inocula distributed to many more plates. For both methods, growth at antimicrobial concentrations below MPC is confirmed by retesting colonies for growth on agar containing the selecting concentration of drug. To assure that the mutants are stable, they are grown on drug-free agar prior to retesting **(28).**

Development of de novo Resistance

Demonstration of the mutant selection window in terms of pharmacokinetic profiles provides a framework for considering initial stages in the development of resistance. Antimicrobials are usually administered to produce tissue concentrations above the MIC. This allows time for host defenses to reduce the pathogen population to where bacterial outgrowth and disease symptoms do not occur after treatment is stopped. When defense systems are inadequate, drug action is required to eliminate the pathogen. Drug costs and potential side effects tend to keep concentrations low while still providing a favorable patient outcome. However, hundreds, and perhaps thousands of resistant cells can be present prior to administration of antibiotic (mutation frequencies are often in the order of 10^6 to 10^8, whereas bacterial infections can contain 10^{10} organisms). Doses of antimicrobial that are inside the selection window can allow growth of the mutant portion of the population **(24).**

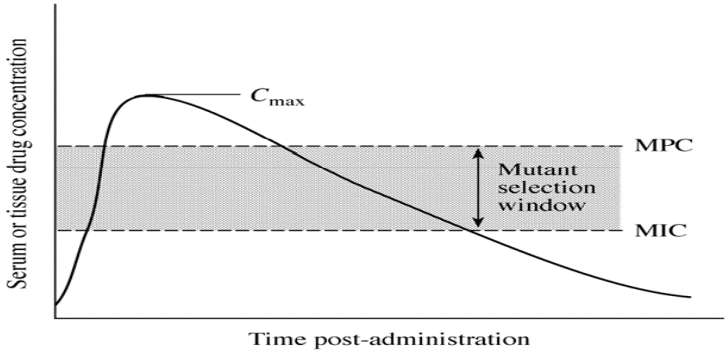

Figure 3. Pharmacodynamic demonstration of the mutant selection window. A hypothetical pharmacokinetic profile is shown in which MIC and MPC are arbitrarily indicated. Double-headed arrow indicates the mutant selection window **(24).**

When episodes of infection are brief, mutant enrichment in an individual patient may not be detected easily. Nevertheless, passage of a pathogen through many treated patients is expected to increase the mutant fraction of the bacterial population gradually. Even if infection arises from a single pathogen cell, over time the probability increases for a given cell to be resistant. Thus an antimicrobial agent may cure 99% of the cases, but when millions are considered, the development of resistance is an inevitable consequence of dosing strategies that place drug concentrations inside the mutant selection window. Two general scenarios can lead to de novo resistance. In the first, the presence of more than one mutation is required for a cell to be considered resistant. With this pattern, resistant populations develop stepwise through the gradual accumulation of mutations that individually reduce susceptibility by low-to-moderate increments. When individual cells are tested, some are found to have intermediate levels of susceptibility. An example of this pattern is the development of fluoroquinolone resistance in Streptococcus pneumoniae: recent clinical isolates of S. pneumoniae contain a variety of target and non-target resistance mutations **(29).**

If a strain already contains a resistance mutation, the next mutational step is achieved more readily. Thus, the development of resistance accelerates with the accumulation of mutations. Since many alleles can accumulate, incremental improvements in fluoroquinolone activity are likely to be neutralized by selective enrichment of mutants. The second scenario is illustrated by treatment of Mycobacterium tuberculosis with most agents, and treatment of Escherichia coli and S. aureus with rifampicin. In these situations, antimicrobial resistance arises in a single step—a mutation reduces susceptibility so much that no tolerable concentration of drug can block mutant growth. In this situation, the upper boundary of the window (MPC) is above the maximum tolerable drug concentration; individual organisms in a bacterial population are either very susceptible or highly resistant **(30).**

Most plasmid-borne resistance is expected to fall in the single-step category, since many rounds of selective pressure are likely to have occurred prior to plasmid entry into the bacterial population in question. According to the data given by Drlica and Zhao about mutant selection window (MSW) and mutant prevention concentration (MPC), maintaining drug concentrations above its MPC throughout therapy can severely restrict the acquisition of drug resistance and achieve its therapeutic effect, while this will increase the risk of adverse and toxic effects. Simultaneously, the drug concentration will unavoidably fall into its MSW **(24)**

Inspired by the analyses of combination therapies reported we empirically deduced that synergistic validity was a key to prevent antimicrobial resistance and balance these factors during combination therapy. Theoretically, the more remarkable the synergistic effect of two antimicrobial agents in a combination was, the more probable their MSWs were to close each other. Thereby, we may discover synergistic combination closing each other's MSWs to avoid their drug concentrations falling into its MSW as possible as we can, and obtain content therapeutic effect with lower dose **(11).**

Antimicrobial Combinations

As there are increasing numbers of reports on bacterial resistance to antibiotics especially during monotherapy, new therapeutic strategies are urgently required to combat MDR Gram negative bacteria and there is a renewed interest towards combination therapy. The co-administration of two or more drugs (combination therapy) is a promising approach, especially if the drugs exhibit synergy, **i.e.**, enhanced efficacy over the predicted additive effects **(31).**

Purpose

The initial use of combination therapy for infections with Gram negative bacteria is justified by one of the following reasons:

(i) to broaden the empiric coverage provided by two antimicrobial agents with different spectra of activity (an effort to ensure that the pathogen is adequately covered by at least one of the two components of the regimen),

(ii) to achieve the synergy observed in vitro between two antibiotic agents compared to one (and hence improve clinical outcomes),

(iii) to allow the administration of lower doses of each antimicrobial in order to reduce their toxicity.

(iv) to prevent or delay the emergence of resistance during antimicrobial therapy **(32).**

Suggested antibiotic combinations

Clinical data to support the choice of antibiotic combinations are sparse and conflicting. Outcome might be difficult to be assessed for the severely ill patients because of frequent changes in antibiotic therapy, co-morbidity, and high all-cause mortality. Moreover, the results for specific combinations might differ between studies because of differences in patient material, infections, antibiotics used, dosage regimens, treatment durations, and strain-dependent factors. Combination therapy for suspected Gram-negative infection includes a broad-spectrum β-lactam and an aminoglycoside or a fluoroquinolone. The lack of effective antibiotics against CR Gram negative bacteria and the drying out of the pipeline of new antibiotics forced physicians to recall old antibiotics (ie, polymyxins) back into clinical use. Most CR Gram negative bacteria, including those with XDR profile, show in vitro susceptibility to colistin. However, the possibility of emergence of hetero-resistance and the low clinical efficacy of colistin in monotherapy in addition to its high toxicity, led to the utilization of colistin in combination with other antibiotics **(33)**

Colistin/carbepenem combination has been commonly used in treatment of CR Gram negative bacteria. Colistin disrupts the bacterial outer membrane facilitating the entry of carbapenem that acts on inhibition of bacterial wall synthesis. The addition of rifampin to colistin and

meropenem/doripenem has resulted in synergistic in vitro effects against MDR Pseudomonas, Acinetobacter and carbapenemase producing Enterobacterales **(34).**

Colistin based combinations (e.g., with rifampicin, carbapenems, ampicillin/sulbactam, Fosfomycin and tigecycline) have been successful against MDR Acinetobacter species . Also, the use of co-delivered combinations of colistin and ciprofloxacin is a promising approach for the treatment of MDR P. aeruginosa infections, particularly in pulmonary delivery for lung infections **(16).** Triple colistin- based combinations appear to be more active than double combinations and more likely to prevent regrowth during treatment and prevent the emergence of resistant subpopulations **(35).**

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